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This is the first year of this three year IDEA Award. The most challenging aspect of this project is the isolation of novel cDNAs encoding human homologs of yeast DNA damage response genes. To date complementation of the yeast mutant *rad9* have not yielded human cDNAs with significant homology. Thus, over the last year we have made a major effort to isolate cDNAs by degenerate PCR strategies which is still underway. In addition, the use of specific two hybrid screens using known human DNA damage response/cell cycle genes has resulted in the isolation of human homologs of *RAD18* and *RAD21*. The subsequent objectives are focused on determination of whether cDNAs isolated in genetic screens are altered in expression or structure in breast cancers. The reagents including RNA, DNA and protein from human breast cancer cell lines grown under identical culture conditions were produced during the first year of the companion CDA award. This set of reagents has been used and demonstrated increased expression of the *RAD21* RNA in some breast cancer cell lines. This prompted the development of a Rad21 antibody which has been completed and will be used for analysis of protein expression in these lines and murine models of mammary tumorigenesis in subsequent years of the grant.

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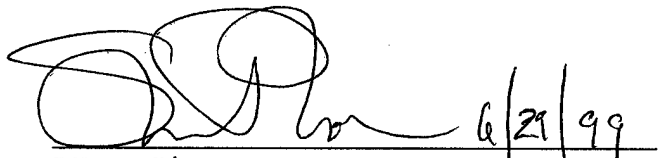

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Molecular Basis of the Response to Radiation Therapy

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Introduction

The goal of this project is to further define at a molecular level the human gene products required for the normal G2 checkpoint response after DNA damage. The checkpoint response is a fundamental mechanism by which cells control their cell division cycle after experiencing DNA damage from radiation. This response results in an arrest in the G2 phase of the cycle until damage is repaired. This checkpoint response is conserved among eukaryotes including the budding yeast *Saccharomyces cerevisiae*. In our application, we proposed to exploit this conservation to isolate human checkpoint genes by large scale complementation screens and homology searches isolating novel human cDNAs which can complement yeast G2 checkpoint mutant strains. Subsequent Technical Objectives are directed towards understanding the structure and expression of these genes in both normal and malignant mammary cells including human cell lines and murine models of mammary tumorigenesis. We also planned to perform functional assays of these cDNAs in checkpoint deficient cell lines including the MCF-7 human breast cancer cell line. In this report we detail progress in the first year of this award towards all three objectives. This grant is co-funded with the companion Career Development Award for the PI, Dr. Sharon Plon (grant #DAMD17-97-1-7284).

A. Progress toward completing the proposed Technical Objectives.

Materials and Methods:

Complementation Assay: Exponential cultures of a *cdc9-8, rad9 strain* grown in YM-1 media were transformed with purified cDNA library DNA using a modified Li-Acetate transformation protocol of Schiestl, and Giets and yeast total RNA and denatured salmon sperm DNA as carrier to achieve a transformation efficiency of 300,000 colonies per microgram plasmid DNA. After transformation the plates were incubated at 23°C overnight. In the morning plates were transferred to 30°C and incubated for 5 days. Colonies growing at this point are isolated and restreaked on leucine deficient media at 30°C for further analysis.

PCR Conditions and Analysis: Degenerate primers are designed after doing alignment of yeast checkpoint genes using the ClustalW program on the BCM Human Genome Project Search Launcher. PCR conditions are optimized for both Mg^{++} concentration and annealing temperature with control templates (either cloned *S. cerevisiae* genes or genomic samples from *S. cerevisiae* and *S. pombe*). After PCR amplification of human cDNA libraries as described below, PCR products are analyzed on 4% NuSieve agarose gels to allow size discrimination of small PCR products.

PCR products of appropriate sizes are subcloned using either the Invitrogen TA or Topo cloning kits. Inserts are confirmed by miniprep DNA preparations and restriction digest. Representative clones for each size are then sequenced using dideoxy automated sequencing methodologies (Licor or ABI automated sequencers).

Two-hybrid Reagents: Reagents used in the two-hybrid screening include the Gal4-activation domain (AD) library, the Gal4-DNA binding (DB) vector (pPC97), the yeast host strain MV103 (Mat **a**, *leu2*, *trp1*, *his3*, Gal1:HIS3, Gal1:LacZ, Spal:URA3), and 5 constructs in MV103 for use as reference controls during screening (22, 23). Control plasmids include 1) DB-pPC97+AD-pPC86, 2) DB-pRb+AD-E2F1, 3) DB-Fos+AD-Jun, 4) Gal4+AD, 5) DB-dDP-1+AD-dE2F.

Activation domain-cDNA library: A human T-lymphocyte cDNA fusion library in the activation domain vector pPC86 (Trp+) was kindly provided by J. La Baer (MGH Cancer Center). The cDNAs were cloned into the *EcoRI* (5') and *SpeI* (3') sites. This library has approximately $2X10^6$ clones and the average insert size is 1kb. This library was amplified once by electroporation using electrocompetent *E. coli*, JS4 cells (BioRad, Hercules, CA) followed by replica plating onto LB+Ampicillin plates. The DNA was prepared using a Plasmid Maxi kit from Qiagen.

Selection of interacting genes: The bait (Leu+) and the library plasmid (Trp+) were sequentially transformed into the yeast host strain MV103 . The transformants containing the bait and library plasmids were selected on media lacking leucine and tryptophan. Three separate pools of library DNA were used to transform the MV103+pPC97-*CDC34* or MV103+pPC97-*RAD6* cells and 500,000 transformants from each pool were obtained. The two-hybrid screen was performed by first selecting for growth of bait-library co-transformants on Sc-His-Leu-Trp+25mM 3AT. Subsequently additional reporter genes, *URA3* and *LacZ*, were selected for in the 3AT positive clones. The expression of the *URA3* gene was both selected for on media lacking uracil as well as counterselected against on media containing uracil and 0.1% 5-fluoroorotic acid (5FOA). Induction of the *LacZ* gene was assayed qualitatively in the presence of X-Gal for blue colonies. The phenotypes were then scored. Clones positive for all the reporters were PCR cloned into a pPCRII vector using TA-cloning kit from Invitrogen (Torry Pines, CA). Both strands of the DNA were then sequenced using a LI-COR automated sequencer.

RESULTS

Technical Objective 1 - Isolation of additional human G2 checkpoint genes.

a. **Complementation Assay:** During the first year of the companion CDA award a large-scale complementation screen of a *rad9,cdc9-8* strain was performed as described. Fifteen human cDNAs were isolated as part of that screen. During this year further characterization of those fifteen cDNAs was performed. A test for plasmid dependence including isolating the human cDNA containing plasmid from the yeast transformant was performed with subsequent re-transformation of the *rad9, cdc9-8* strain. This analysis revealed that fourteen of the clones did not confer plasmid dependence, eg, were due to reverting mutations in the strain. For the fifteenth strains, the plasmid did confer partial rescue of the checkpoint defect. The cDNA inserts were subcloned into sequencing vectors. Sequence analysis of this clone revealed a partial cDNA encoding a ribosomal protein that did not reveal any homology to *RAD9* protein. Thus, we were not able to identify an active cDNA through this complementation assay.

b. A major emphasis during this year of the grant was the use of homologous regions between evolutionarily distant species (*S. cerevisiae and S. pombe)* to develop degenerate PCR based primers. For example, a fission yeast homolog of *RAD9* named *rhp9* was published. Alignment of those sequences reveals areas of homology that may suggest conserved regions of the protein. One such area is in the carboxy terminus consistent with the known BRCT domain. In addition there are more discreet areas of homology in the carboxy terminus

that may reflect *RAD9/rhp9* specific conserved domains. We have made a major effort to develop a series of degenerate PCR primers to this regions. Overall over 200 PCR reaction sets (varying primers and templates) have been performed. These primers are optimized on test templates including the *RAD9* gene itself as well as yeast genomic DNA. We have now begun amplification from human cDNA sources including lymphocyte cDNA libraries. We have also done amplification from Lamprey DNA which represents a species that is approximately intermediate in evolution between humans and yeast. The PCR products are then subcloned and sequenced as described in the method section. Table 1 illustrates the number of PCR primers developed, the number of fragments subcloned and sequenced. To date the sequences obtained do not demonstrate addition regions of homology to the *S. cerevisiase RAD9* gene. An analogous approach has been taken with *DUN1* and to date subcloned PCR products from human lymphocyte cDNA libraries has not yielded homologous sequences.

Table 1 – Summary of Degenerate PCR approach to cloning of Human *RAD9* and *DUN1*

Gene	Primer Sets	Templates	Clones Sequenced
RAD9/rhp9	4	Human T cell library (3 independent pools) Mouse Embryonic library Lamprey Genomic DNA	19
DUN1	4	Human T cell library (3 independent pools) Lamprey Genomic DNA	38

Our analysis of why we have not obtained sequences lead to three potential possibilities (1) such homologous sequences do not exist in the human genome, (2) the cDNA libraries chosen do not represent tissue where the gene is expressed and (3) the general bias towards shorter cDNAs in library constructs may discriminate against the generally long checkpoint cDNAs. Given that we find the first possibility unlikely based on the overall conservation of the pathway and the conservation of rad9 and rhp9, we are addressing the second two concerns. We have obtained human cDNA from multiple sources including normal mammary gland, an explant of a human breast carcinoma, normal ovarian tissue and bone marrow in order to maximize the chance of finding a tissue specific gene. The use of cDNA will avoid bacterial contamination seen in library samples and provide detection of sequences from long cDNAs. We are currently using two sets of degenerate primers for the *RAD9* homologs on these sets of cDNAs. Any PCR products of appropriate length will be subcloned and sequenced. If additional regions of homology to *RAD9* are identified then this sequence will be used as a probe to identify longer cDNAs. This same approach is now being taken with three

sets of degenerate primers for homologous regions of *DUN1* and the human cDNA samples.
A third approach is searching of human cDNA and genomic sequence databases. We have recently identified a short human EST cDNA which is a partial clone with a 200 amino acid open reading frame with homology to *S. cerevisiae RAD9*. We are using this cDNA to identify other anonymous cDNAs from this locus, will build a computer-based contig of the sequence as well as isolate longer cDNAs from this locus. Our goal is to determine if this partial cDNA represents a portion of a longer cDNA with significant homology to *RAD9*.

c. Several other genetic screens including two-hybrid screens in yeast for human cDNAs in the DNA damage checkpoint and repair response have been accomplished. As part of those screens cDNAs encoding the human homolog of *S. pombe RAD21* and *S. cerevisiae RAD18* were isolated. The *RAD21* sequence has been previously reported in the literature although the human gene has not been thoroughly characterized previously (see below). The *RAD18* sequence has not been reported. We are currently screening additional cDNA libraries to obtain a full length clone of this gene for subsequent analysis in aims 2 and 3..

Technical Objective 2A – Checkpoint gene structure and expression in human breast cancer cell lines.

As described in the original grant application and begun during year one of the CDA award, we obtained normal human mammary epithelial cells (HMEC) from Clonetics and a panel of eight human mammary derived cell lines, MCF10A, MCF7, MDA-MB-157, MDA-MD-231, MDA-MB-136, BT-20, HBL100 and SKBR-3. These were all grown in culture under controlled conditions. All the cell lines were grown in the same DFCI media to minimize artifacts due to culture technique including both the immortal but not transformed MCF10 and the transformed lines. One exception is the HMEC cultures which require a separate proprietary media. In parallel RNA, DNA and protein lysates were derived from these cultures. The isolation of RNA was repeated on a fresh set of cultures in order to be able to replicate any findings on the first set. Analysis of expression of *RAD21* reveals increased expression at the RNA level in breast cancer cell lines in comparison to the HMEC controls.

In order to pursue that finding with regard to human *RAD21* RNA we created reagents to perform detailed analysis of the Rad21 protein. We have identified an antigenic peptide in the carboxy terminus based on homology with the antibody previously raised against the sea urchin protein. This peptide was synthesized and used in production of an anti-human Rad21 polyclonal antibody. ELISA analysis of this serum shows high reactivity. In addition Western blot analysis demonstrates that the antisera recognizes a protein of the appropriate molecular weight in lysates from human cell lines with very high affinity. In addition in order to be able to perform analysis of immunoprecipated protein (using a polyclonal IP, monoclonal Western method) we have also begun development of an anti-human Rad21 monoclonal antibody. A fusion

construct with human Rad21 and GST tag was constructed and used for the production of the antigen. Production of the monoclonal antibody is underway.

Technical Objective 3 - Determination of Changes in Response to Radiation of a Human Breast Cancer Cell Line upon Expression of Human Checkpoint Genes.

The first portion of this aim is based on examining whether the human cDNAs previously identified or identified in Aim 1 will suppress the G2 checkpoint defect in the human MCF-7 breast cancer cell line. We have begun this analysis for human Rad21 using an epitope tagged version. With development of the antibodies as described in aim 2 this analysis will be further facilitated. Analysis of ectopically expressed Rad21 by Western blot does not show significant differences in the level of the protein after exposure to UV and gamma irradiation. Alternative gel techniques will need to be used in order to determine if there is a change in phosphorylation or nuclear localization after DNA damage.

Key Research Accomplishments:

- Analysis of human cDNAs obtained by screening of a human cDNA library for complementation of *rad9* mutant strain completed.
- Extensive degenerate PCR cloning to obtain human homologs of *RAD9* and *DUN1* initiated.
- Cloning of cDNAs encoding the human homolog of *RAD21* and *RAD18*.
- Polyclonal antibody to human Rad21 protein produced.

Reportable Outcomes:

- Work on *CHES1* was presented as an invited talk at the International Meeting on Fork head/ Winged Helix Proteins at Scripps Research Institute, La Jolla CA, November 1999.
- Polyclonal antibody to human Rad21 protein produced.

C. Conclusions

In the first year of this three year IDEA Award we have made progress all three Technical Objectives. The most challenging aspect of this project is the isolation of novel cDNAs encoding human homologs of yeast DNA damage response genes. To date complementation of the yeast mutant *rad9* have not yielded human cDNAs with significant homology. Thus, over the last year we have made a major effort to isolate cDNAs by degenerate PCR strategies which is still underway. Finally, we do routine searches of human cDNA and genomic databases for newly sequenced genes which demonstrate homology (we are currently following up on a new EST with homology to *RAD9*). We will continue over the next year to focus on these latter two strategies for both human homologs of *RAD9* and *DUN1*. In addition, the use of specific two hybrid screens using known human DNA damage response/cell cycle genes has resulted in the isolation of human homologs of *RAD18* and *RAD21*.

The subsequent objectives are focused on determination of whether cDNAs isolated in genetic screens are altered in expression or structure in breast cancers. The reagents including RNA, DNA and protein from human breast cancer cell lines grown under identical culture conditions were produced during the first year of the CDA award. This set of reagents has been used and demonstrated increased expression of the *RAD21* RNA. This prompted the development of a Rad21 antibody which has been completed and will used for analysis of protein expression in these lines and murine models of mammary tumorigenesis. Similarly, in Objective Three this antibody will be used to further probe alteration in Rad21 expression or modification after DNA damage. In addition to analysis of genes isolated by this investigator a number of other groups have published cDNA sequences for human homologs of checkpoint genes including Rad53. These genes will be incorporated into our analysis of regulation in normal and malignant mammary cells in the next year of this award.